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Tbp2 FRAGMENTS OF THE TRANSFERRIN RECEPTOR OF  
NEISSERIA MENINGITIDIS

The present invention relates to polypeptides derived from the Tbp2 subunit of the transferrin receptor of *Neisseria meningitidis*, to their use in a therapeutic capacity, in particular for vaccination, as well as to the DNA fragments coding for these polypeptides.

Generally speaking, meningitis is either of viral origin or of bacterial origin. The bacteria mainly responsible are *N. meningitidis* and *Haemophilus influenzae*, which are involved, respectively, in approximately 40 and 50 % of cases of bacterial meningitis.

Approximately 600 to 800 cases per annum of *N. meningitidis* meningitis are recorded in France. In the United States, the number of cases amounts to approximately 2,500 to 3,000 per annum.

The species *N. meningitidis* is subdivided into serogroups according to the nature of the capsular polysaccharides. Although a dozen serogroups exist, 90 % of cases of meningitis are attributable to 3 serogroups: A, B and C.

Effective vaccines based on capsular polysaccharides exist to prevent meningitis caused by *N. meningitidis* serogroups A and C. These polysaccharides as such display little or no immunogenicity in children under 2 years of age and do not induce immune memory. However, these drawbacks may be overcome by conjugating these polysaccharides to a carrier protein.

In contrast, the polysaccharide of *N. meningitidis* group B displays little or no immunogenicity in man, irrespective of whether or not it is in conjugated form. Thus, it is seen to be highly desirable to seek a vaccine against meningitis induced by *N. meningitidis* in particular of serogroup B other than a polysaccharide-based vaccine.

To this end, different proteins of the outer membrane of *N. meningitidis* have already been proposed. In this connection, special attention has been directed towards the membrane receptor for human transferrin.

Generally speaking, the large majority of bacteria need iron for their growth, and they have developed specific systems for acquiring this metal. As regards *N. meningitidis* in particular, which is a strict pathogen of man, the iron may be taken only from human iron-transport proteins such as transferrin and lactoferrin, since the amount of iron in free form is negligible in man (of the order of  $10^{-18}$  M), in any case insufficient to permit bacterial growth.

Thus, *N. meningitidis* possesses a receptor for human transferrin and a receptor for human lactoferrin which enable it to bind these iron-chelating proteins and thereafter to take up the iron needed for its growth.

The transferrin receptor of *N. meningitidis* strain B16B6 has been purified by Schryvers et al. (WO 90/12591) from a membrane extract. This protein as purified is seen to consist essentially of 2 types of polypeptide: a polypeptide with a high apparent molecular weight of 100 kD and a polypeptide with a lower apparent molecular weight of approximately 70 kD, as visualized after polyacrylamide gel electrophoresis in the presence of SDS.

The product of the purification carried out, in particular, by Schryvers is, by arbitrary definition and for the purposes of the present patent application, referred to as the transferrin receptor, and the polypeptides constituting it, as subunits. In the text which follows, the subunits of high molecular weight and of lower molecular weight are referred to, respectively, as Tbp1 and Tbp2.

Furthermore, since the pioneering work of Schryvers et al., it has been discovered that there are in fact, at least 2 types of strain which differ in the constitution of their respective transferrin receptors. This was demonstrated by studying membrane extracts of several tens of strains of *N. meningitidis* of miscellaneous origins. These membrane extracts were first subjected to polyacrylamide gel electrophoresis in the presence of SDS, and then electrotransferred onto

nitrocellulose membranes. These nitrocellulose membranes were incubated:

- a) in the presence of a rabbit antiserum directed against the transferrin receptor purified from *N. meningitidis* strain B16B6, also referred to as IM2394;
- b) in the presence of a rabbit antiserum directed against the transferrin receptor purified from *N. meningitidis* strain M982, also referred to as IM2169; or
- c) in the presence of human transferrin conjugated to peroxidase.

As regards a) and b), the recognition of the transferrin receptor subunits is visualized by adding an anti-rabbit immunoglobulin antibody coupled to peroxidase, and then by adding the substrate for this enzyme.

Tables I and II below show the profile of some representative strains as appears on 7.5 % polyacrylamide gel after electrophoresis in the presence of SDS; the bands are characterized by their apparent molecular weights expressed in kilodaltons (kD):

	Strains		
Table I	2394 (B;2a;P1.2:L2,3) 2228 (B; nd) 2170 (B;2a:P1.1:L3)	2234 (Y; nd) 2154 (C; nd) 2448 (B; nd)	550 (C;2a:) 179 (C;2a:P1.2)
Detection with anti-2394 receptor antiserum	93 68	93 69	99 69
Detection with anti-2169 receptor antiserum	93	93	99
Detection with peroxidase-transferrin	68	69	69

NB: In brackets, the serogroup, serotype, subtype and immunotype are shown in order.

	Strains								
Table II	2169 (B:9:P1.9)	1000 (B:nd)	1604 (B:nd)	132 (C:15:P1.16)	1001 (A:4:P1.9)	876 (B:19:P1.6)	1951 (A:nd)	2449 (B:nd)	867 (B:2b:P1.2)
Detection with anti-2394 receptor antiserum	96	98	98	98	98	96	94	94	93
Detection with anti-2169 receptor antiserum	96	98	98	98	98	96	94	94	93
Detection with peroxidase- transferrin	87	85	83	81	79	88	87	85	85

NB: In brackets, the serogroup, serotype, subtype and immunotype are shown in order.

The results entered on the first 2 lines of the tables show that there are 2 types of strain:

The first type (Table I) corresponds to strains which possess a receptor both of whose subunits are recognized, under the experimental conditions used, by the anti-IM2394 receptor antiserum, while only the high molecular weight subunit is recognized by the anti-IM2169 receptor antiserum.

The second type (Table II) corresponds to strains which possess a receptor both of whose subunits are recognized, under the experimental conditions used, by the anti-IM2169 receptor antiserum, while only the high molecular weight subunit is recognized by the anti-IM2394 receptor antiserum.

Consequently, an antigenic diversity exists in respect of the lower molecular weight subunit. This diversity is, however, restricted since it resolves into 2 major types, contrary to the suggestion made by Griffiths et al., FEMS Microbiol. Lett. (1990) 69: 31.

In conformity with this, reference will be made in the text which follows to IM2169 type or IM2394 type strains.

Besides the strains mentioned in Table II, IM2169 type strains are, for example, the strains S3032 (12, P 1.12.16), 6940 (19, P 1.6), M978 (8, P 1.1, 7), 2223 (B: nd), 1610 (B: nd), C708 (A: 4, P 1.7), M981 (B: 4), also referred to as 891, and 2996 (B: 2b, P 1.2). The Applicant received the free gift of the strains S3032, M978 and M981 from Dr. J. Poolman (RIVM, Bilthoven, Holland), and the strain C708 from Dr. Achtman (Max Planck Institute, Berlin, Germany).

The strain IM2154 (serogroup C) is mentioned by way of example as being of the IM2394 type.

On the basis of the above findings, the assumption was possible that a vaccine which is effective against all *N. meningitidis* infections could be adequately composed of the high molecular weight subunit, irrespective of the strain of origin of the receptor, since this subunit is recognized by both types of

antiserum. However, it seems that this cannot be the case, inasmuch as the high molecular weight subunit appears to be incapable of inducing the production of neutralizing type antibodies. Only the smaller of the two  
5 receptor subunits (Tbp2) appears to be capable of performing this function.

The amino acids sequences of the Tbp2 subunits of the strains IM2169 and IM2394 have been disclosed in Patent Application EPA 586,266 (published on 9th March  
10 1994), as well as the corresponding DNA fragments. These sequences are repeated in SEQ ID NO 1 to 4 of the present application.

In SEQ ID NO 5 to 10, the sequences of the Tbp2 subunits of the IM2169 type strains, namely the strains  
15 M978, 6940 and S3032, are presented.

It is shown, furthermore, that the sequence of the IM2154 (IM2394 type) Tbp2 subunit differs by two amino acids from the sequence of the IM2394 Tbp2 subunit, at positions 306 and 510.

It has now been found that a Tbp2 subunit, irrespective of the strain of origin, possesses in structural terms three main domains, at least one of which is associated with special properties. By definition, the domains of IM2169 Tbp2 and IM2394 Tbp2  
20 have been fixed as shown in the table below, by indicating the position of the amino acids, limits of the different domains included, and by reference to the numbering appearing in SEQ ID NO 1 and 3.

	Tbp2 Im2169	Tbp2 IM2394
30 N-terminal domain or first domain	1-345	1-325
Hinge domain or second domain	346-543	326-442
C-terminal domain or third domain	544-691	443-579

35 This definition applies similarly to all IM2169

or IM2394 type Tbp2's, after alignment of an IM2169 or IM2394 type sequence with the reference sequence, to maximum homology. Thus, by way of example and by reference to Figure 1, the position is shown of the domains of the Tbp2 subunit of M978 as follows: first domain (1-346), second domain (347-557) and third domain (558-705).

Furthermore, it was also found that the N-terminal domain or first domain and/or the hinge domain or second domain could be necessary and sufficient for the purpose of inducing a vaccinal effect in humans; accordingly, it would not be essential to use a Tbp2 in a complete form. It was found in particular that the first domain contained the transferrin binding site virtually in its entirety, was hence very probably exposed to the outside and consequently constituted a component of choice for vaccinal purposes.

Lastly, it was found that certain regions of the second domain of the IM2169 type Tbp2's were quite generally variable and immunodominant. Two approaches are hence possible with a view to a vaccine; either it is considered that the immunodominant epitopes may mask other epitopes of vaccinal interest, and they are consequently deleted, or use is made of this variability in order to retain only these regions in a vaccine.

Accordingly, the invention provides a polypeptide having an amino acid sequence which is derived from that of a Tbp2 subunit of the transferrin receptor of an IM2169 or IM2394 type *N. meningitidis* strain whose first, second and third domains are defined by alignment to maximum homology with the sequence of the Tbp2 subunit of the respective reference strain, IM2169 or IM2394; in particular by total or partial deletion of at least one domain of the said IM2169 or IM2394 type Tbp2 subunit, provided the first and second domains are not totally deleted simultaneously.

"Sequence which is derived from another sequence" is obviously understood to mean a sequence originating from this other sequence by the use of a mental process.

More especially, a polypeptide according to the

invention possesses an amino acid sequence which is derived from an IM2169 or IM2394 type Tbp2 subunit:

- 5 (i) in particular by total or partial deletion of at least one domain of the said Tbp2 subunit, selected from the second and third domains; preferably by total or partial deletion of the third domain or of the second and third domains;
- 10 (ii) in particular by total deletion of the first and third domains, or
- (iii) in particular by complete deletion of the third domain and by partial deletion of the first domain, optionally by partial deletion of the second domain.

*Ins.*  
*C4* 15 *C4* Advantageously, a polypeptide according to the invention has a partial, virtually total or total deletion of the third domain, preferably a total deletion. In this case, the first and also the second domain may be maintained in their entirety, partially or totally  
20 deleted, independently of one another.

The following combinations are possible (given that the first, second and third domains in their entirety are represented by 1, 2 and 3, respectively, and that  $\circ$  and  $\Delta$  mean partially and totally deleted, respectively):  
25

1, 2,  $\Delta$ 3; 1,  $\circ$ 2,  $\Delta$ 3; 1,  $\Delta$ 2,  $\Delta$ 3;  
 $\circ$ 1, 2,  $\Delta$ 3;  $\circ$ 1,  $\circ$ 2,  $\Delta$ 3;  $\circ$ 1,  $\Delta$ 2,  $\Delta$ 3;  
 $\Delta$ 1, 2,  $\Delta$ 3;  $\Delta$ 1,  $\circ$ 2,  $\Delta$ 3;

30 1, 2,  $\circ$ 3; 1,  $\circ$ 2,  $\circ$ 3; 1,  $\Delta$ 2,  $\circ$ 3;  
 $\circ$ 1, 2,  $\circ$ 3;  $\circ$ 1,  $\circ$ 2,  $\circ$ 3;  $\circ$ 1,  $\Delta$ 2,  $\circ$ 3;  
 $\Delta$ 1, 2,  $\circ$ 3;  $\Delta$ 1,  $\circ$ 2,  $\circ$ 3;

Interest also attaches to a polypeptide according to the invention derived from an IM2169 type Tbp2 subunit by partial deletion of the second domain, which  
35 contains in their entirety or virtual entirety the first and third domains; that is to say the combination 1,  $\circ$ 2,



3. ("Domain maintained in its virtual entirety" is understood here and in the text which follows to mean a domain modified at a very small number of positions, not more than 5 approximately.) A polypeptide according to the invention can also correspond to the combination 01, 02, 3, the partial deletion of the first domain advantageously affecting the region homologous to that of IM2169 Tbp2 extending from the amino acid at position 1 to the amino acid approximately at position 40.

10 When a polypeptide according to the invention is derived, in particular, by partial deletion of the second domain of an IM2169 type Tbp2 subunit, this partial deletion advantageously affects one or more regions of the second domain which is/are the homologue(s) of the regions of the IM2169 sequence extending:

- (i) from the amino acid at position 362 to the amino acid at position 379;
- (ii) from the amino acid at position 418 to the amino acid at position 444;
- 20 (iii) from the amino acid at position 465 to the amino acid at position 481; and
- (iv) from the amino acid at position 500 to the amino acid at position 520.

25 Preferably, the partial deletion simultaneously affects the four regions (i) to (iv) described above.

When a polypeptide according to the invention is derived, in particular, by complete deletion of the third domain and virtually complete deletion of the second domain of an IM2169 type Tbp2 subunit and contains the whole of the first domain, or is derived, in addition, by deletion of the N-terminal portion of the first domain, the virtually complete deletion of the second domain extends over the region which:

- in the case of a polypeptide derived from an IM2169 type Tbp2 subunit, is the homologue of the region of the second domain of the IM2169 Tbp2 subunit extending from the amino acid in one of the positions 346 to 361 to the amino acid at position 543;

5       - in the case of a polypeptide derived from an IM2394 type Tbp2 subunit, is the homologue of the region of the second domain of the IM2394 Tbp2 subunit extending from the amino acid in one of the positions 326 to 341 to the amino acid at position 442.

10       When a polypeptide according to the invention is derived, in particular, by partial deletion of the first domain of an IM2169 or IM2394 type Tbp2 subunit, this partial deletion advantageously affects all or part of the region:

15       (i) which is the homologue of the region of the first domain of the said IM2169 type Tbp2 subunit extending from the amino acid at position 1 to the amino acid at position 281; or

20       (ii) which is the homologue of the region of the first domain of the said IM2394 type Tbp2 subunit extending from the amino acid at position 1 to the amino acid at position 266.

By way of example of the foregoing, a deletion of interest affecting the region:

25       (i) which is the homologue of the region of the first domain of the said IM2169 type Tbp2 subunit extending from the amino acid at position 1 to the amino acid approximately at position 40; or

30       (ii) which is the homologue of the region of the first domain of the said IM2394 type Tbp2 subunit extending from the amino acid at position 1 to the amino acid approximately at position 45

may be mentioned.

35       The IM2169 or IM2394 type sequence from which that of a polypeptide according to the invention is derived displays a degree of homology with the respective reference sequence, IM2169 or IM2394, advantageously of at least 70-75 %, preferably of at least 80 %, and, as a more special preference, of at least 90 %.

According to a most especially preferred embodiment, a polypeptide according to the invention possesses a sequence derived from that of the IM2169 or IM2394 Tbp2 subunit.

5           The degree of homology may be readily calculated by aligning the sequences so as to obtain the maximum degree of homology; to this end, it may be necessary to introduce vacant positions artificially, as illustrated in Figures 1 to 4 and 8 to 10. Once optimum alignment is  
10 achieved, the degree of homology is established by reckoning up all the positions in which the amino acids of the two sequences turn out to be identical, relative to the total number of positions.

          It would be tedious to described homologous  
15 sequences other than in a generic manner, on account of the excessively large number of combinations. A person skilled in the art knows, however, the general rules which enable one amino acid to be replaced by another without abolishing the biological or immunological  
20 function of a protein.

          By way of a preferred example, a polypeptide according to the invention whose sequence possesses at least 70-75 %, advantageously at least 80 %, preferably at least 90 %, and, as an absolute preference, 100 %,  
25 homology with:

- (i)       the sequence as shown in ID SEQ NO 1, from the amino acid at position 1 to the amino acid at position 345;
- (ii)      the sequence as shown in ID SEQ NO 3, from  
30 the amino acid at position 1 to the amino acid at position 325 or 442;
- (iii)     the sequence as shown in ID SEQ NO 1, from the amino acid at position 1 to the amino acid at position 691 or 543, from which the  
35 regions 362-379, 418-444, 465-481 and 500-520 have been deleted;
- (iv)      the sequence as shown in ID SEQ NO 1, from the amino acid at position 346 to the amino acid at position 543,

may be mentioned.

Polypeptides corresponding to the definition given in the paragraph above are illustrated as follows:

- 5 (i) A polypeptide according to the invention whose sequence is substantially as shown in ID SEQ NO 1, 5, 7, 9, 36 or 38, from the amino acid at position 1 to the amino acid at position 350, 351, 354, 358, 322 or 346, respectively;
- 10 (ii) A polypeptide according to the invention whose sequence is substantially as shown in ID SEQ NO 3, from the amino acid at position 1 to the amino acid at position 330;
- 15 (iii) A polypeptide according to the invention whose sequence is substantially as shown in:
- ID SEQ NO 1, from the amino acid at position 1 to the amino acid at position 691, from which the regions 362-379, 418-444, 465-481 and 500-520 have been deleted;
  - 20 - ID SEQ NO 5, from the amino acid at position 1 to the amino acid at position 705, from which the regions 365-382, 421-453, 474-495 and 514-534 have been deleted;
  - ID SEQ NO 7, from the amino acid at position 1 to the amino acid at position 693, from which the regions 366-383, 422-448, 469-485 and 504-524 have been deleted;
  - 25 - ID SEQ NO 9, from the amino acid at position 1 to the amino acid at position 699, from which the regions 372-389, 428-454, 475-491 and 510-529 have been deleted;
  - 30 - ID SEQ NO 36, from the amino acid at position 1 to the amino acid at position 699, from which the regions 339-356, 395-421, 443-458 and 477-497 have been deleted;
  - 35 - ID SEQ NO 38, from the amino acid at position 1 to the amino acid at position 699, from which the regions 363-380, 429-

445, 467-482 and 501-521 have been deleted; and

(iv) A polypeptide according to the invention whose sequence is substantially as shown in:

- 5                   - ID SEQ NO 1, from the amino acid at position 346 to the amino acid at position 543,
- ID SEQ NO 5, from the amino acid at position 347 to the amino acid at position 557,
- 10                  - ID SEQ NO 7, from the amino acid at position 350 to the amino acid at position 557,
- ID SEQ NO 9, from the amino acid at position 354 to the amino acid at position 551,
- 15                  - ID SEQ NO 36, from the amino acid at position 323 to the amino acid at position 521, or
- 20                  - ID SEQ NO 38, from the amino acid at position 345 to the amino acid at position 544.

25                   Particular polypeptides corresponding to the definitions in points (i) to (iv) are described in the examples which follow.

30                   A polypeptide according to the invention possesses an amino acid sequence which comprises at least 10, advantageously at least 20, preferably at least 50, and, as an absolute preference, at least 100, amino acids.

35                   Obviously, a polypeptide according to the invention can also comprise, additionally, an amino acid sequence which does not display homology with the sequences of the Tbp2 subunits of strains IM2169 and IM2394; which sequences are shown in ID SEQ NO1 and 3 from the amino acid at position 1 to the amino acid at the C-terminal position.

                  Generally speaking, an additional sequence can be that of any other polypeptide excluding Tbp2.

For example, an additional sequence can be that of a signal peptide localized at the N-terminal position of a polypeptide according to the invention. Examples of a signal sequence are shown in ID SEQ NO 1 to 4. Furthermore, it may be pointed out that a suitable heterologous signal sequence can be a signal sequence of a gene coding for a lipoprotein.

The subject of the invention is also:

- (i) an isolated DNA fragment coding for a polypeptide according to the invention;
- (ii) an expression cassette which comprises at least one DNA fragment according to the invention, placed under the control of elements capable of providing for its expression in a suitable host cell; and
- (iii) a method of production of a polypeptide according to the invention, according to which a host cell containing an expression cassette according to the invention is cultured.

"Isolated DNA fragment" means that a DNA fragment according to the invention is not integrated in a DNA fragment coding for a complete Tbp2 subunit.

In the expression cassette, the DNA fragment according to the invention can be combined or otherwise with a DNA block coding for a signal peptide which is heterologous or otherwise to the polypeptide encoded by the said DNA fragment, depending on whether or not secretion of the polypeptide is sought. Preferably, this secretion will be sought.

Elements such as a DNA block coding for a heterologous signal peptide (signal region) or a promoter already exist in quite large numbers, and are known to a person skilled in the art. His general competence will enable him to choose a particular signal region or promoter which will be suited to the host cell in which he envisages expression.

For the purposes of the method according to the invention, the host cell can be a mammalian cell, a

bacterium or a yeast, the latter two being preferred. Here too, the choice of a particular line is within the capacity of a person skilled in the art.

The invention also relates to a monoclonal  
5 antibody:

- (i) capable of recognizing an epitope present in the first domain of an IM2169 or IM2394 type Tbp2 subunit; the said epitope having a sequence homologous to that present in the  
10 first domain of the Tbp2 subunit of the strain IM2394 and selected from YKGTW (SEQ ID NO 32), EFEVDFSDKTIKGTI (ID SEQ NO 33), EGGFYGPKGEEL (ID SEQ NO 34) and AVFGAK (ID SEQ NO 35); and optionally,
- 15 (ii) incapable of recognizing the epitope present in the third domain of the said IM2169 or IM2394 type Tbp2 subunit, whose sequence is homologous to that of the epitope of the first domain which is recognized.

20 In order to illustrate the above point (ii), it may be pointed out by way of example that the sequences of the third domain of the IM2394 Tbp2 subunit which are homologous, two by two, to those of the first domain occur at positions 443-447, 472-485, 537-548 and 568-573,  
25 respectively.

Preferably, a monoclonal according to the invention is:

- (i) capable of recognizing the region present in the first domain of an IM2169 or IM2394 type  
30 Tbp2 subunit whose sequence is homologous to the sequence EGGFYGPKGEEL present in the first domain of the Tbp2 subunit of the strain IM2394; and optionally,
- 35 (ii) incapable of recognizing the epitope present in the third domain of the said IM2169 or IM2394 type Tbp2 subunit, an epitope equivalent to the one which is recognized, whose sequence is homologous to the sequence SGGFYGKNAIEM present in the third domain of the Tbp2 subunit of the

strain IM2394.

A preferred monoclonal is:

- (i) capable of recognizing the epitope GFYGPK present in the first domain of a Tbp2 subunit of the strain IM2394; and
- (ii) incapable of recognizing the equivalent epitope present in the third domain of the said IM2394 Tbp2 subunit.

In effect, such a monoclonal has been recognized as bactericidal, and consequently it is possible to envisage using it as active principle in a pharmaceutical composition in passive immunotherapy to combat an *N. meningitidis* infection.

Lastly, the invention also relates to a pharmaceutical composition comprising as active principle at least one polypeptide according to the invention.

A pharmaceutical composition according to the invention is, in particular, useful for inducing an immune response in humans against *N. meningitidis*, inter alia a vaccinal effect so as to protect humans against *N. meningitidis* infections, in prevention or in therapy.

A composition according to the invention advantageously comprises as active principle at least two polypeptides according to the invention; that is to say at least one first polypeptide whose sequence is derived from that of an IM2169 type Tbp2 subunit, and at least one second polypeptide whose sequence is derived from that of an IM2394 type Tbp2 subunit. Alternatively, a composition according to the invention can also contain at least one polypeptide whose sequence is derived from that of an IM2169 type Tbp2 subunit and at least one IM2394 type Tbp2 subunit.

As regards the IM2394 type polypeptide, a component of the pharmaceutical composition, it is greatly preferable for this polypeptide to contain all or part of the sequence which is homologous to that of the first domain of the IM2394 Tbp2 subunit from which it is derived. The portion of the sequence which should preferably be maintained is the homologue of the region of the



IM2394 Tbp2 subunit extending from the amino acid at position 267 to the amino acid at position 325. The sequence of such a polypeptide can be derived from that of an IM2394 type Tbp2 subunit, in particular by total or partial deletion of the region of the second or third domain of the IM2394 type Tbp2 subunit.

Thus, for the purposes of a pharmaceutical composition containing two types of component (IM2394 type and IM2169 type), the following IM2394 type polypeptides are more especially preferred:

1, 2, O3; 1, 2, Δ3; 1, O2, Δ3; 1, Δ2, Δ3  
O1, 2, O3; O1, 2, Δ3; O1, O2, Δ3; O1, Δ2, Δ3.

As regards the IM2169 type polypeptide, a component of the pharmaceutical composition, two preferred approaches are possible:

(A) - Either to combine with the IM2394 type polypeptide a polypeptide which contains all or part of the sequence which is homologous to that of the first domain of the IM2169 Tbp2 subunit from which it is derived. In this case, the portion of the sequence which should preferably be maintained is the homologue of the region of the IM2169 Tbp2 subunit extending from the amino acid at position 282 to the amino acid at position 345. The sequence of such a polypeptide may be derived from that of an IM2169 type Tbp2 subunit, in particular by total or partial deletion of the region of the second or third domain of the IM2169 type Tbp2 subunit.

Thus, according to this alternative and for the purpose of a pharmaceutical composition containing two types of component (IM2394 type and IM2169 type), the following IM2169 type polypeptides are more especially preferred:

1, 2, O3; 1, 2, Δ3; 1, O2, Δ3; 1, Δ2, Δ3  
O1, 2, O3; O1, 2, Δ3; O1, O2, Δ3; O1, Δ2, Δ3.

1, O2, 3; O1, O2, 3.

As regards the last two possibilities (1, 02, 3; 01, 02, 3), the partial deletion of the second domain may very advantageously affect one or more regions of the second domain which is/are the homologue(s) of the regions of the IM2169 sequence extending:

- (i) from the amino acid at position 362 to the amino acid at position 379;
- (ii) from the amino acid at position 418 to the amino acid at position 444;
- 10 (iii) from the amino acid at position 465 to the amino acid at position 481; and
- (iv) from the amino acid at position 500 to the amino acid at position 520.

Preferably, the partial deletion simultaneously affects the four regions (i) to (iv) described above.

(B) - Or to combine with the IM2394 type polypeptide a polypeptide whose sequence is derived by partial deletion of the second domain and by total or virtually total deletion of the first or third domain of the IM2169 type Tbp2 subunit, and contains the second domain in its entirety ( $\Delta 1$ , 2,  $\Delta 3$ ). In this alternative, the pharmaceutical composition containing two types of component (IM2394 type and IM2169 type) may advantageously contain several IM2169 type polypeptides ( $\Delta 1$ , 2,  $\Delta 3$ ); for example two or more of the polypeptides selected from ( $\Delta 1$ , 2,  $\Delta 3$ ) IM2169, M978, 6940 and S3032.

A pharmaceutical composition according to the invention may be manufactured in a conventional manner. In particular, the polypeptide(s) according to the invention is/are combined with an adjuvant, a diluent or a vehicle which is acceptable from a pharmaceutical standpoint. A composition according to the invention may be administered by any conventional route in use in the vaccine field, especially subcutaneously, intramuscularly or intravenously, for example in the form of an injectable suspension. The administration can take place in a single dose or a dose repeated one or several times after a certain time interval. The appropriate dosage varies in accordance with various parameters, for example the

individual being treated or the mode of administration.

In order to establish the subject of the present invention, it may be pointed out that the *N. meningitidis* strains IM2394 and IM2169 are available to the public from the Collection Nationale de Culture des Micro-organismes [National Collection of Microorganism Cultures] (CNCM), Pasteur Institute, 25 rue du Dr Roux 75015 Paris under the respective registration numbers LNP N 1511 and LNP N 1520.

The invention is described in greater detail in the examples below and by reference to Figures 1 to 10.

~~Figures 1 to 3, 8 and 9 present, respectively,~~ the alignments of the M978, 6940, S3032, BZ83 and BZ163 Tbp2 sequences with the IM2169 Tbp2 sequence, to maximum homology. The respective degrees of homology are 78.9, 81.2, 79.6, 71.3 and 81.8 %.

Figure 4 presents the alignments to maximum homology of the sequences of the hinge domains (second domain) of IM2169 (1), 6940 (2), 2223 (3), C708 (4), M978 (5), 1610 (6), 867 (7), S3032 (8) and 891 (9) Tbp2. The numbering of IM2169, as it appears in ID SEQ NO 2, is given in italics. The sequences which may be deleted according to a preferred embodiment appear in bold type. (C) indicates the consensus sequence.

Figures 5 to 7 illustrate the construction of the plasmids pTG5782, pTG5755 and pTG5783, respectively.

Figure 10 presents the alignments to maximum homology of the sequences of the hinge domains (second domain) of IM2169 (1), 2223 (2), 708 (3), M528 (4), 6940 (5), M978 (6), 1610 (7), S3032 (8), 867 (9), BZ83 (10) and BZ163 (11) Tbp2. The numbering of IM2169, as it appears in ID SEQ NO 2, is given in italics. The sequences which may be deleted according to a preferred embodiment appear in bold type. (C) indicates the ~~consensus sequence.~~

EXAMPLE 1 Polypeptide T/2169 (1, O2, Δ3; 1-350) including the sequence as shown in ID SEQ NO 1 (IM2169), from the amino acid at position 1 to the amino acid at position 350

**1A - Preparation of the DNA fragment coding for T/2169 (1-350):**

### Construction of the vector pTG 5782.

From the plasmid pTG3721 described in Application  
5 EPA 586,266, a *Hind*III restriction site is introduced by  
site-directed mutagenesis downstream of the sequence  
coding for Tbp2, to generate the plasmid pTG4704.

From plasmid pTG3721, a fragment containing the sequence coding for the secretion signal of RlpB and from the beginning of the sequence coding for mature Tbp2 up to the internal *Hae*II site is amplified by PCR using the primers OTG4915 and OTG4651.

OTG4915 : AAACCCGGATCCGTTGCCAGCGCTGCCGT  
HaeII

(SEQ ID NO: 21)

OTG4651:

*Bsp*HI

TTTTTTCATG AGA TAT CTG GCA ACA TTG TTG TTA TCT CTG

Met Arg Tyr Leu Ala Thr Leu Leu Leu Ser Leu

GCG GTG TTA ATC ACC GCC GGG TGC CTG GGT GGC  
Ala Val Leu Ile Thr Ala Gly Cys Leu Gly ...  
                    cleavage of the signal peptide

GGC GGC AGT TTC

15           The PCR fragment is then digested with *Bsp*HI and  
HaeII and inserted simultaneously with the HaeII-*Hind*III  
fragment of pTG4704, which contains the 3' portion of the  
region coding for Tbp2, into the plasmid pTG3704  
described in Application EPA 586,266, digested with *Nco*I  
20 and *Hind*III, to generate the plasmid pTG5768.

From plasmid pTG3721, a fragment containing the sequence coding for the N-terminal portion of Tbp2 is amplified by PCR using the primers OTG4928 and OTG5011.

(SEQ ID NO: 22)

SphI

OTG4928 : GTG TTT TTG TTG AGT GCA TGC CTG GGT GGC  
Val Phe Leu Leu Ser Ala Cys Leu Gly Gly

- cleavage of the  
signal peptide

(SEQ ID NO: 23)

OTG5011 : TGCGCAAGCTTACAGTTTGTCTTTGGTTTTTCGCGCTGCCG  
HindIII

This PCR fragment is digested with SphI and HindIII and then cloned into the plasmid pTG4710 described in Application EPA 586,266; the plasmid pTG5740 is thereby generated.

- 5 The HaeII-HindIII fragment of pTG5740, containing the 3' portion of the sequence coding for the human transferrin (hTf) binding domain (3' of the region coding for the first domain), is inserted into plasmid pTG3704 digested with BamHI and HindIII, simultaneously with the
- 10 BamHI-HaeII fragment of pTG5768 containing the araB promoter, the rlpB signal sequence and the beginning of the coding sequence of Tbp2; the plasmid pTG5782 is thereby generated. This vector contains the araB promoter, and the sequence coding for the secretion signal of
- 15 RlpB fused to the sequence coding for the N-terminal domain of Tbp2 (1-350).

#### 1B - Production and purification of T/2169 (1-350)

- An *E. coli* strain (Xac-I) is transformed with pTG5782. The transformants are cultured at 37°C in M9
- 20 medium + 0.5 % succinate + 50 µg/ml arginine + 100 µg/ml ampicillin. In the exponential phase, 0.2 % of arabinose (inducer) is added. After one hour of induction, cells are removed and extracts are prepared. Western blot analysis followed by visualization with hTF-peroxidase
- 25 enables a preponderant band to be detected, whose MW corresponds to that expected for this truncated form of Tbp2.

In a test as described in Example 4 of WO93/6861 (published: 15.04.93), purified T/2169 proves capable of

inducing bactericidal antibodies, and should consequently be useful for vaccinal purposes.

EXAMPLE 2: Polypeptide T/2394 (1, O2, Δ3; 1-340) including the sequence as shown in ID SEQ NO 2 (IM2394), from the amino acid at position 1 to the amino acid at position 340.

**2A - Preparation of the DNA fragment coding for T/2394 (1-340):**

**Construction of the vector pTG 5755**

From the plasmid pTG4710 described in Application EPA 586,266, a fragment containing the region coding for the C-terminal portion of the hTf binding domain is amplified by PCR using the primers OTG4873 and OTG4877. This fragment is then digested with MluI and HindIII.

(SEQ ID NO: 24)  
OTG4873 : AAAAAGCATGCATAAAACTACGCGTTACACCATTCAAGC

(SEQ ID NO: 25)

OTG4877 : TATATAAGCTTACGTTGCAGGCCCTGCCGCGTTTCCCC  
HindIII

Plasmid pTG4710 is digested with MluI and HindIII. The MluI-HindIII fragment containing the 3' portion of the sequence coding for Tbp2 is replaced by the PCR fragment coding for the C-terminal portion of the hTf binding domain. The plasmid pTG5707 is thereby generated. A BamHI-MluI fragment containing the araB promoter and the beginning of the sequence coding for Tbp2 is then replaced, in plasmid pTG5707, by a BamHI-MluI fragment of pTG4764 described in Application EPA 586,266, which contains the araB promoter, and the sequence coding for the RlpB secretion signal fused to the sequence coding for the N-terminal domain of Tbp2. The plasmid pTG5755 is thereby generated. This vector contains the araB promoter, and the sequence coding for the secretion signal of RlpB fused to the sequence coding for the N-terminal domain of Tbp2 (1-340).

**2B - Production and purification of T/2394 (1-340)**

T/2394 (1-340) is produced and purified as described in Example 1B.

In a test as described in Example 4 of WO93/6861 (published: 15.04.93), purified T/2394 proves capable of inducing bactericidal antibodies, and should consequently be useful for vaccinal purposes.

**EXAMPLE 3:** Polypeptide D4/2169 (1, 02, 3) whose sequence is identical to that as shown in ID SEQ NO 1, from the amino acid at position 1 to the amino acid at position 691, from which the regions 362-379, 418-444, 465-481 and 500-520 have been deleted.

**3A - Preparation of the DNA fragment coding for D4/2169****1.1. Cloning of the DNA fragment.**

The DNA fragment coding for the Tbp2 subunit of *N. meningitidis* strain IM2169 is amplified by PCR (polymerase chain reaction) using specific primers complementary to the 5' and 3' regions (A5' and A3', respectively), on 10 ng of genomic DNA extracted from a culture of bacteria of the strain IM2169.

(SEQ ID NO: 26)  
A5' : 5' CCCGAATTCTGCCGTCTGAAGCCTTATTC 3'  
(SEQ ID NO: 27)  
A3' : 5' CCCGAATTCTGCTATGGTGCTGCCTGTG 3'

A DNA fragment is thereby obtained and, after digestion with *EcoRI*, it comprises 2150 nt. This *EcoRI* fragment is then ligated to the dephosphorylated *EcoRI* ends of the phagemid pBluescriptSK(-) (Stratagene) to give the recombinant phagemid pSK/2169tbp2.

**1.2. Implementation of the deletions.**

The clone pSK/2169tbp2 containing the *tbp2* sequences of the strain M982 is deleted by the technique of Kunkel, PNAS (1985) 82: 448.

In brief, the phage form of the recombinant phagemid pSK/2169tbp2 is obtained after rescue with the helper phage VCS M13 according to the technique described by Stratagene, the supplier of the parent vector, and

used to infect the bacterial strain CJ236. The outcome of the *dut* and *ung* mutations carried by strain CJ236 is the synthesis of DNA molecules which have incorporated the nucleotide precursor dUTP.

- 5           The phages are harvested and the single-stranded DNA is extracted with a phenol/chloroform mixture. This DNA is hybridized under standard conditions with the following oligonucleotides:

(SEQ ID NO: 28)

C  
C  
C  
(SEQ ID NO: 29) 2169d1 : 5' CGCATCCAAAACCGTACCTGTGCTGCCTGA 3'  
(SEQ ID NO: 30) 2169d2 : 5' TTTATCACTTTCCGGGGGCAGGAGCGGAAT 3'  
(SEQ ID NO: 31) 2169d3 : 5' GTTGGAACAGCAGACAGCGTTTGCGCCCC 3'  
(SEQ ID NO: 32) 2169d4 : 5' GAACATACTTTGTTCGTTTTTGC GCGTCAA 3'

- 10           The hybridization reaction is continued for 30 min at a temperature decreasing from 70°C to 30°C.

The complementary second strand is then completed by total synthesis in the presence of the four deoxynucleotides, T4 DNA polymerase and T4 DNA ligase, according to standard conditions.

- 15           The strain *E. coli* SURE (Stratagene) is transformed with the DNA thereby obtained. In this strain, the molecules carrying dUTP, that is to say unmutated molecules, are destroyed.

- 20           The phages obtained are analysed by the standard techniques of rapid preparation of plasmid DNA and of digestion with the appropriate restriction enzymes. The presence of the desired mutation is then verified by nucleotide sequencing.

- 25           The clone pSK2169#7, carrying the four mutations Δ 1203-1256, Δ 1371-1451, Δ 1512-1562 and Δ 1617-1679, is selected.

### 3B - Construction of the expression vector pTG5783

- 30           Plasmid pTG5768 described above is digested with *HpaI* and *XcmI*. An *XcmI*-*XcmI* fragment of pTG5768 and the *HpaI*-*XcmI* fragment of plasmid pSK/2169ed#7 are inserted simultaneously into this vector to generate the plasmid pTG5783. This vector contains the *araB* promoter, and the sequence coding for the secretion signal of RlpB fused to



the modified *tpb2* sequence (deletions d1 to d4).

### 3C - Preparation and purification of D4/2169.

D4/2169 is produced and purified according to Example 1B.

5 In a test as described in Example 4 of WO93/6861 (published: 15.04.93), purified D4/2169 proved capable of inducing bactericidal antibodies, and should consequently be useful for vaccinal purposes.

10 EXAMPLES 4 to 8: Polypeptides 4) C/2223, 5) C/M981, 6) C/1610, 7) C/M978 and 8) C/C708 corresponding to the second domain (hinge region) of Tbp2's of various strains.

15 The DNA fragments coding for the Tbp2's of *N. meningitidis* strains 2223, M981, 1610, M978 and C708 were cloned by PCR amplification as described in Example 3A, using the same two primers. Similarly, these fragments were inserted at the *EcoRI* or *EcoRI/BamHI* sites of the phagemid pBluescriptSK(-). Sequencing of the region  
20 coding for the second domain was performed, and the amino acid sequence deduced as each one of them is seen in Figure 4.

On the basis of each of the nucleotide sequences, primers specific to each of the two domains are created  
25 by introducing suitable cleavage sites for the purpose of a future cloning in frame with *rlpB* signal sequence, under the control of the *araB* promoter. These primers are used in PCR to amplify the region coding for the second domain of each of the Tbp2's. These regions are cloned as  
30 described above into a plasmid containing the *rlpB* signal sequence, under the control of the *araB* promoter.

Expression of the peptides is carried out as described in Example 1B.

35 EXAMPLE 9: Vaccine composition (T/2169-T/2394) intended for preventing *N. meningitidis* infections

Sterile solutions of T/2169 and T/2394 as purified in Examples 1B and 2B are thawed. In order to prepare one litre of vaccine containing 100 µg/ml of each of the active principles, the following solutions are mixed

under sterile conditions:

- Solution of T/2394 containing 1 mg/ml  
in buffer C (500 mM phosphate buffer,  
pH 8, 0.05 % Sarkosyl) 100 ml
- 5 - Solution of T/2169 containing 1 mg/ml  
in buffer C 100 ml
- Buffered physiological solution (PBS)  
pH 6.0 300 ml
- Aluminium hydroxide containing 10 mg  
10 Al<sup>+++</sup>/ml 50 ml
- 1 % (w/v) merthiolate in PBS 10 ml
- PBS qs 1,000 ml

EXAMPLE 10: Vaccine composition (D4/2169-Tbp2/2394).  
intended for preventing *N. meningitidis*  
15 infections

A sterile solution of D4/2169 as purified in  
Example 3C is thawed. A sterile solution of Tbp2/2394 as  
prepared and purified in Example 3 of EPA 586,266 is  
treated similarly. In order to prepare one litre of  
20 vaccine containing 100 µg/ml of each of the active  
principles, the following solutions were mixed under  
sterile conditions:

- Solution of Tbp2/2394 containing 1 mg/ml  
in buffer C 100 ml
- 25 - Solution of D4/2169 1 mg/ml in buffer C 100 ml
- Buffered physiological solution (PBS)  
pH 6.0 300 ml
- Aluminium hydroxide containing 10 mg  
Al<sup>+++</sup>/ml 50 ml
- 30 - 1 % (w/v) merthiolate in PBS 10 ml
- PBS qs 1,000 ml

EXAMPLE 11: Obtaining an antibody capable of recognizing  
the epitope GFYGPKE of the first domain of  
IM2394 Tbp2.

- 35 11A - Immunization of mice and production of hybridomas  
MRL/Lpr-Lpr mice, known to produce more IgG2a,  
IgG2b and IgG3 than Balb/C mice (J. Immunol. Methods  
(1991) 144: 165), receive a first intraperitoneal injec-  
tion of 50 µg of the IM2394 membrane fraction in the

presence of Freund's complete adjuvant. The membrane fraction which is used is prepared as follows:

The strain IM2394 stored in lyophilized form is taken up and cultured on Mueller-Hinton agar overnight at 37°C in an atmosphere containing 20 % of CO<sub>2</sub>. The layer is taken up and is used to inoculate an Erlenmeyer containing Mueller-Hinton broth to which 30 µM EDDA (ethylenediaminedi(ortho-hydroxyacetic acid) - Sigma) has been added. After 5 hours of incubation at 37°C with rotary stirring, the culture is centrifuged. The pellet is taken up with Tris-HCl buffer, pH 8, and the suspension is lysed in an ultrasound apparatus operating at high pressure (Rannie, model 8.30H). The suspension obtained is centrifuged at low speed to remove cell debris and the membranes are collected by ultracentrifugation (140,000 xg, 75 min, 4°C). The membrane fraction is finally taken up in 50 mM Tris-HCl buffer, pH 8, and its protein concentration determined.

This first injection is followed by two booster injections 21 and 49 days later. The booster doses contain 25 µg of the Tbp2 protein as purified in Example 3 of EPA 586,266, in the form of an emulsion in Freund's incomplete adjuvant.

56 days later, the mouse which has developed the highest antibody titre (monitoring of immune sera by ELISA) is selected for the production of specific monoclonal antibodies. This mouse receives a final booster injection (78 days after the initial injection), inoculating 25 µg of the Tbp2 protein as purified in Example 3 of EPA 586,266, both intravenously and intraperitoneally. 3 days later, the animal's spleen is removed and the spleen cells are fused with P3 x 63 Ag 8653 mouse myeloma cells in a ratio of one myeloma cell to 4 spleen cells. The fusion protocol used is derived from that described initially by G. Köhler and C. Milstein, Nature (1975) 256: 495. After fusion, the cells are arranged in sterile microwells (Nunc) coated with a nutrient "feeder", on the basis of 100,000 cells per well in a volume of 200 µl of selective medium [DMEM medium containing 20 % of FCS and

a hypoxanthine/azaserine/thymidine mixture at a concentration of 2 % (V/V) (Gibco. Ref 043-01060H)]. The selective medium is replaced 6 days later by a non-selective medium [DMEM medium containing 20 % of FCS and a hypoxanthine/thymidine mixture at a concentration of 2 % (V/V) (Gibco. Ref 043-01065H)].

#### 11B - Screening of the hybridomas

The culture supernatants of the hybridomas are tested by ELISA according to the following method:

10 Into ELISA plate microwells "sensitized" overnight at +4°C with 100 µl of a solution containing 5 µg/ml of RT 2394 in carbonate buffer (50 mM pH 9.6) and then saturated for 1 hour at 37°C with 200 µl of a 0.1 M phosphate buffer containing 1 % of bovine serum albumin (weight/volume) (PBS-BA), there are introduced 100 µl of hybridoma culture supernatant (or dilutions of immune sera prepared in PBS-BA buffer containing 0.05 % of Tween 20) (PBS-T-BA). After a further incubation for 1 h 30 min at 37°C followed by 5 washes in PBS-Tween, the wells are coated with 100 µl of a mixed solution of antibodies conjugated to alkaline phosphatase (AP) which are specific for the mouse isotypes IgG<sub>2a</sub>, IgG<sub>2b</sub> and IgG<sub>3</sub>, so as to select only hybridomas secreting specific antibodies which are functional in the test of bactericidal effect.

25 The mixed solution of conjugated antibodies is prepared by diluting the following 3 goat immune sera, namely goat anti IgG<sub>2a</sub>-AP (Caltag), goat anti IgG<sub>2b</sub>-AP (Caltag) and goat anti IgG<sub>3</sub>-AP (Caltag), to 1/1500 in PBS-T-BA buffer. After incubation of the solution of conjugated antibodies for 1 h 30 min at 37°C followed by 5 washes, the enzyme reaction is visualized with 100 µl of a solution of paranitrophenyl phosphate containing 5 mg/ml in 0.1 M diethanolamine buffer, pH 9.8. The development of the reaction is stopped after 30 min by adding 50 µl of 1N sodium hydroxide, before spectrophotometric analysis at 405 nm.

35 Clones which are positive after this first screening are analysed for their capacity to recognize the Tbp2 subunit by Western blotting.

To this end, the transferrin receptors of IM2394

(0.863 mg/ml) and of IM2169 (0.782 mg/ml) as prepared in Examples 1 and 2 of WO93/6861 are diluted to 1/10 in 1M Tris buffer, pH 6.8, and then denatured by adding 10 % (V/V) of a 25 % solution of SDS in TE buffer (100 mM Tris-HCl, 10 mM EDTA), pH 8.0, and 5 % (V/V) of  $\beta$ -mercaptoethanol. After treatment for 15 min at 56°C, a 110  $\mu$ l aliquot containing the denatured IM2394 or IM2169 transferrin receptor is applied to a 7.5 % polyacrylamide gel. After migration (1 hour at 200 volts in a Biorad cell), the proteins are electrotransferred onto a nitrocellulose membrane (100 volts for 50 min). The membrane is saturated overnight at room temperature in 20 mM Tris buffer, 137 mM NaCl, pH 7.6 (TBS) containing 5 % (W/V) of skimmed milk powder, then mounted on a miniblottter. The antibodies which are tested are adjusted to a concentration of 25  $\mu$ g/ml in TBS buffer containing 1 % (W/V) of milk powder before being applied in the proportion of 50  $\mu$ l per channel.

After 45 min of incubation, followed by rinses in TBS buffer/1 % milk, 50  $\mu$ l of a rabbit anti-mouse IgG.A.M immune serum (Zymed) conjugated to alkaline phosphatase and diluted beforehand 1000 times in TBS buffer/1 % milk are applied to each channel.

After a further incubation for 45 min, followed by rinses, the enzyme reaction is visualized using a chromogenic substrate (BCIP/NBT (Sigma Fast R)). The reaction is stopped after 15 min by dipping into distilled water. Positive clones are characterized by their capacity to disclose a band corresponding to a protein of approximately 69 kD (Tbp2 subunit) after electrotransfer of the IM2394 transferrin receptor onto a nitrocellulose membrane.

At the end of this second screening by Western blotting, the clones are analysed for their capacity to produce an immunoglobulin which reacts with the peptide sequence GFYGPKE in an ELISA system; the methodology is identical to that described above, except for the sensitization of the plates, which is carried out by adding 100  $\mu$ l of a solution of peptide GFYGPKE containing

2 µg/ml into each well.

Among the hybridomas tested, one which proves capable of reacting with the peptide is selected; it is then stabilized by successive clonings (at least 2) on the basis of 5 cells/well during the first cloning and one cell/well during subsequent clonings.

#### 11C - Production and purification of the monoclonal antibody

The monoclonal antibody is produced in ascites of male nude Swiss mice.

15 days after injection of 500 µl of pristane intraperitoneally, the nude mice receive a second intraperitoneal injection of 7 million cells originating from the hybridoma.

The ascites fluids are withdrawn under sterile conditions and then purified by affinity chromatography on a column of G protein. The ascites diluted to 1/5 in 0.1M phosphate buffer, pH 7.4, and filtered through a 0.22 µ Millipore filter is passed through a column G protein previously equilibrated in the same phosphate buffer, at the rate of 40 ml/hour.

The antibodies bound to the column are eluted using 0.1M glycine buffer, pH 2.7. The eluted fractions are immediately neutralized using 1 M Tris buffer pH 8.0 (in the proportion of 1 volume of Tris to 10 volumes of eluate).

The eluate is then dialysed overnight at +4°C in 0.1M phosphate buffer, pH 7.4, aliquoted and stored frozen.

The purity of the antibody is monitored by electrophoresis on 7.5 % polyacrylamide gel and by permeation chromatography on Superose 12. The degree of purity is generally greater than 95 %.

By applying the protocol described above and screening approximately 800 hybridomas, a monoclonal capable of reacting with the epitope GFYGPKEGE of the first domain of IM2394 Tbp2, and incapable of reacting with the corresponding epitope located in the third domain (that is to say GFYGKNAI), was selected in par-

(SEQ ID NO: 43)

(SEQ ID NO: 44)

ticular.

This monoclonal (referred to as 475 E<sub>7</sub>) is an IgG<sub>2b</sub> of isoelectric point between 7.8 and 8.1, and possesses a bactericidal titre of 512.

5 This titre was determined as follows:

From a solution of Mab 475 E<sub>7</sub>, doubling dilutions are made and incubated in the presence of 50 µl of a suspension of meningococci containing 1x10<sup>4</sup> CFU/ml and 50 µl of young rabbit complement [the bacterial  
10 suspension is obtained by culturing *N. meningitidis* strain B16B6 at 37°C for 5 hours in Mueller-Hinton-Difco broth containing 30 µM EDDA (ethylenediaminedi(ortho-hydroxyphenylacetic acid) - Sigma)].

After one hour of incubation at 37°C, 25 µl of  
15 mixture are withdrawn and cultured on supplemented Mueller-Hinton agar. The agar dishes are incubated overnight at 37°C under an atmosphere containing 10 % of CO<sub>2</sub>. The colonies are counted and the bactericidal titre is expressed as the reciprocal of the final dilution in the  
20 presence of which 50 % or more lysis of the bacteria is observed relative to the control.

Under these conditions, it was determined that the Mab 475 E<sub>7</sub> possessed a bactericidal titre of 512.

EXAMPLE 12: Demonstration of the bactericidal activ-  
25 ity of immunoglobulins specific for the protein T/2169 (1-350) with respect to various *N. meningitidis* strains.

12A - Production and purification of T/2169 (1-350)

A strain of *E. coli* B is transformed with plasmid  
30 pTG5782 described in Example 1. The transformant selected is amplified to give seeding batches. From a tube of *E. coli* B transformed with pTG 5782, amplification of the culture is carried out in M9 medium + 0.5 % succinate. Culturing is carried out in a 20-l fermenter.

35 In the exponential phase, arabinose (expression inducer) is added. After one hour of incubation, the cells are harvested and ruptured in an apparatus operating at high pressure (Rannie) and the membrane fraction is harvested by centrifugation.

Western blot analysis followed by visualization with transferrin-peroxidase enables a preponderant band to be detected whose molecular weight corresponds to that expected for this truncated form. The protein is purified  
5 by preparative SDS-PAGE from 10 % acrylamide gel.

12B - Production of immunoglobulins specific for T/2169  
(1-350)

The protein fraction thereby obtained is used to immunize rabbits. Briefly, rabbits (New Zealand White)  
10 are immunized (i) on D/0 with 50 µg of protein T/2169 prepared as described in 12A, in the presence of Freund's complete adjuvant, and (ii) on D/21 and D/42 with 50 µg of protein T/2169 in the presence of Freund's incomplete adjuvant. On D/56, the rabbits are sacrificed and the  
15 serum is harvested. From this serum, the immunoglobulins are purified by affinity chromatography on a protein A-Sepharose (Pharmacia) resin. The purification is carried out according to the supplier's recommendations. The purified IgG fraction is lyophilized, and the lyophi-  
20 lisate is taken up with a certain volume so that the final protein concentration of the solution is in the region of 25 mg/ml.

12C - Test of bactericidal effect

In parallel with the purification of T/2169, a  
25 purification is carried out by preparative SDS-PAGE of a fraction of *E. coli* B obtained after transformation with the plasmid pTG3704 (this vector is identical to plasmid pTG5782 but does not include any sequence of Tbp2). The protein fraction obtained by preparative SDS-PAGE is used  
30 to immunize rabbits as is described above, and the IgGs are purified from the serum harvested.

Hence two serum fractions designated T/2169 IgG and Control IgG are obtained. They are analysed for their capacity to lyse different strains of *N. meningitidis* in  
35 the test of bactericidal effect, as described in Example 4 of WO 93/6861 (published on 15.04.1993).

The results obtained on different isolates are summarized in the table below, and demonstrate that the



purified protein T/2169 proves capable of inducing antibodies which are bactericidal with respect to several strains of the IM2169 type group. These results of cross bactericidal effect demonstrate that T/2169 should be  
5 useful for vaccinal purposes.

*Determination of the bactericidal activity of immunoglobulins specific for the protein T/2169 in comparison with control immunoglobulins with respect to six N. meningitidis strains*

10	Strain	Serogroup Serotype/subtype	Bactericidal titres*	
			Control IgG	T/2169 IgG
	2169	B:9;P1.9	< 4	128
	RH 873	B;8;P1.1.7	< 4	16
	RH 876	B;19,P1.6	< 4	64
	351	B:NT;P1.7	< 4	256
15	NG G40	B;1:-	< 4	512
	EG 328	B;NT;-	< 4	64

\* Bactericidal titres are expressed as the reciprocal of the dilution for which 50 % lysis of the initial colonies is observed

EXAMPLE 13: Demonstration of the bactericidal activity  
20 of immunoglobulins specific for the protein D4/2169 with respect to various strains of *N. meningitidis*.

#### 13A - Production and purification of D4/2169

D4/2169 is produced and purified according to  
25 Example 12A.

#### 13B - Production of immunoglobulins specific for D4/2169

This production is performed in a similar manner to that described in Example 12B.

#### 30 13C - Test of bactericidal activity

Two immunoglobulin fractions designated D4/2169

IgG and Control IgG are obtained. They are analysed for their capacity to lyse different strains of *N. meningitidis* in the test of bactericidal activity as described in Example 4 of WO 93/6861 (published on 15.04.93).

The results obtained on different isolates are summarized in the table below, and demonstrate that purified D4/2169 proves capable of inducing antibodies which are bactericidal with respect to several strains, and should consequently be useful for vaccinal purposes.

*Determination of the bactericidal activity of immunoglobulins specific for the protein D4/2169 in comparison with control immunoglobulins with respect to six N. meningitidis strains*

Strain	Serogroup Serotype/subtype	Bactericidal titres*	
		Control IgG	D4/2169 IgG
2169	B:9;P1.9	< 4	32
RH 873	B;8;P1.1.7	< 4	8
RH 876	B;19,P1.6	< 4	16
351	B:NT;P1.7	< 4	128
NG G40	B;1:-	< 4	64
EG 328	B;NT;-	< 4	16

\* Bactericidal titres are expressed as the reciprocal of the dilution for which 50 % lysis of the initial colonies is observed.

	SEQ ID NO	Project name	Sequence
	1, 2	IM2169-2	IM2169 Tbp2 complete
	3, 4	IM2394-2	IM2394 Tbp2 complete
	5, 6	M978	M978 Tbp2 complete
5	7, 8	6940	6940 Tbp2 complete
	9, 10	S3032	S3032 Tbp2 complete
	11	2D IM2169	2nd domain of IM2169 Tbp2
	12	2D 6940	2nd domain of 6940 Tbp2
	13	2D 2223	2nd domain of 2223 Tbp2
10	14	2D C708	2nd domain of C708 Tbp2
	15	2D M978	2nd domain of M978 Tbp2
	16	2D 1610	2nd domain of 1610 Tbp2
	17	2D 867	2nd domain of 867 Tbp2
	18	2D S3032	2nd domain of S3032 Tbp2
15	19	2D 891	2nd domain of M981 Tbp2
	20	OTG 4915	OTG 4915
	21	OTG 4651	OTG 4651
	22	OTG 4928	OTG 4928
	23	OTG 5011	OTG 5011
20	24	OTG 4873	OTG 4873
	25	OTG 4877	OTG 4877
	26	A 5'	A 5'
	27	A 3'	A 3'
	28	2169 D1	2169D1
25	29	2169 D2	2169D2
	30	2169 D3	2169D3
	31	2169 D4	2169D4
	32	MAB1	1st box of the 1st domain of IM2169 Tbp2
	33	MAB2	2nd box of the 1st domain of IM2169 Tbp2
30	34	MAB3	3rd box of the 1st domain of IM2169 Tbp2
	35	MAB4	4th box of the 1st domain of IM2169 Tbp2
	36, 37	BZ83	BZ83 Tbp2 complete
	38, 39	BZ163	BZ163 Tbp2 complete

40	2D BZ83	2nd domain of BZ83 Tbp2
41	2D BZ163	2nd domain of BZ163 Tbp2
42	2D M528	2nd domain of M528 Tbp2